Epithelial cell adhesion molecule-targeting designed ankyrin repeat protein-toxin fusion Ec1-LoPE exhibits potent cytotoxic action in prostate cancer cells

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Abstract. Targeted anticancer therapeutics offer the advantage of reducing cytotoxic side effects to normal cells by directing the cytotoxic payload selectively to cancer cells. Designed ankyrin repeat proteins (DARPins) are promising non-immunoglobulin-based scaffold proteins for payload delivery to cancer-associated molecular targets. Epithelial cell adhesion molecule (EpCAM) is overexpressed in 40-60% of prostate cancers (PCs) and is associated with metastasis, increased risk of PC recurrence and resistance to treatment. Here, we investigated the use of DARPin Ec1 for targeted delivery of *Pseudomonas* exotoxin A variant (LoPE) with low immunogenicity and low non-specific toxicity to EpCAM-expressing prostate cancer cells. Ec1-LoPE fusion protein was radiolabeled with tricarbonyl technetium-99m and its binding specificity, binding kinetics, cellular processing, internalization and cytotoxicity were evaluated in PC-3 and DU145 cell lines. Ec1-LoPE showed EpCAM-specific binding to EpCAM-expressing prostate cancer cells. Rapid internalization mediated potent cytotoxic effect with picomolar IC_{50} values in both studied cell lines. Taken together, these data support further evaluation of Ec1-LoPE in a therapeutic setting in a prostate cancer model *in vivo*.

Introduction

Efficient treatment of metastatic prostate cancer (PC) remains a current clinical need (1). Initial androgen deprivation therapy provides stabilization of disease for several years; however, the development of resistance occurs in the large majority of patients with time (2). Insensitivity to existing treatments leads to disease progression, which necessitates the identification of novel molecular targets and targeted therapeutic approaches.

Prostate-specific membrane antigen (PSMA)-targeted therapy using radioligands, such as [^{177}Lu]Lu-PSMA-617, has demonstrated promising results in clinical trials and improved outcomes for patients with metastatic castration-resistant PC (3). However, patients with low PSMA expression are not eligible for this therapy (4); some patients do not respond to it and some develop resistance despite initially good response (5).

Epithelial cell adhesion molecule (EpCAM) is a potential therapeutic target in PC. EpCAM is a type I transmembrane glycoprotein, which participates in cell adhesion and proliferation, and was reported to be involved in oncogenic signaling by engagement of elements of the Wnt pathway (6). Upregulated EpCAM expression is detected in early stages of PC and is further induced in high-grade tumors and metastatic lesions (7,8). Intense EpCAM overexpression is found in 40-60% of prostate tumor samples (9) and is associated with metastasis and increased risk of PC recurrence (10,11). Due to its involvement in mechanisms regulating resistance to treatment, EpCAM has been suggested as a therapeutic target to sensitize PC cells to chemotherapy and radiotherapy (12). The EpCAM protein surface level was found to be up to 16-fold higher in PC cells compared to non-cancerous prostate cells and up to 4-fold higher in malignant compared to benign prostate tumors (7), which creates a potential therapeutic window for targeted cytotoxic delivery.

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A number of therapeutic agents targeting EpCAM have been evaluated in preclinical and clinical trials for different types of cancer (13). They include monoclonal antibodies (mAbs), their fragments, as well as their conjugates with cytotoxic drugs and immunotoxins (14,15). The only EpCAM-targeting agent that has received approval in the European Union is the rat-mouse bispecific anti-EpCAM/anti-CD3 antibody catumaxomab for the treatment of patients with malignant ascites by intraperitoneal infusion (16). While being more effective than conventional treatment, it has shown limited efficacy in solid tumors, which could arise from several factors, such as heterogeneity of EpCAM expression in patients, limited penetration in the tumor due to the size of the targeting agent and limited cytotoxic effect.

One way to increase the potency of targeted therapy is conjugation of a cytotoxic payload, such as drugs, plant or bacterial toxins, to a targeting molecule for selective eradication of malignant cells. The limited therapeutic effect observed for EpCAM-targeting mAbs promoted the development of targeted immunotoxins (17). Several of them have reached clinical trials, such as the mouse mAb MOC31 and its scFv fragment (4D5MOCB) conjugated to *Pseudomonas aeruginosa* exotoxin A (PE) (VB4-845, oportuzumab monatox) (18-21) and a Fab fragment conjugated with the plant-derived ribosome-inactivating protein de-bouganin (VB6-845, citatuzumab bogatox) (22). Still, there is a number of physiological barriers hampering the efficient tumor delivery of cytotoxic payloads using bulky immunoglobulins, and the use of smaller targeting vectors should be beneficial (17).

Designed ankyrin repeat proteins (DARPins) are a type of engineered scaffold proteins with promising tumor-targeting properties (23). Their use for tumor targeting offers a number of advantages in comparison with the traditional IgG scaffold. The small size (14-18 kDa) could enable more efficient interstitial transport, faster extravasation and deeper penetration of DARPins into the tumors compared to therapeutics based on the IgG scaffold (150 kDa) (24). Their high affinity ensures a strong binding to malignant cells and a good retention in tumors. Rapid excretion of DARPins from blood provides high imaging contrast already several hours after injection. DARPins have shown promising results in preclinical studies for imaging of human epidermal growth factor receptor 2 (HER2) (25-28) and EpCAM expression (29-32) and could be used as companion diagnostic agents during targeted therapy. The clinical trial evaluating anti-HER2 DARPin G3 labeled with technetium-99m showed its capacity of specific targeting HER2-positive breast cancers and demonstrated the safety of this protein scaffold in humans, with no toxicity or side effects observed (33).

Another advantage of DARPin scaffold over IgG includes its robustness, high solubility and thermodynamic stability. Conjugates of DARPins with protein-based toxins can be genetically engineered and produced as single protein fusions in large amounts with lower manufacturing costs (13,23,34).

In the present study, we investigated a fusion protein consisting of an EpCAM-targeting DARPin Ec1 and a re-engineered *Pseudomonas* exotoxin A variant (LoPE) bacterial toxin for cytotoxic therapy of prostate cancer. The N-terminal DARPin Ec1 (18 kDa) binds to EpCAM with picomolar affinity [K_D 68 pM (35)], while the C-terminal

LoPE toxin (25 kDa) irreversibly inhibits eukaryotic elongation factor eEF2 that leads to inhibition of protein synthesis in the cell (36). LoPE is the deimmunized C-terminal catalytic subunit of bacterial *Pseudomonas aeruginosa* Exotoxin A (PE toxin) with lower immunogenicity and general toxicity *in vivo* in comparison with previous versions of PE toxin (37).

The aim of the present study was to characterize the functional parameters of Ec1-LoPE, such as binding specificity and kinetics of binding to living cells, cellular processing, internalization and cytotoxicity in PC cell lines and to evaluate its potential for targeted therapy of PC *in vivo*.

Materials and methods

Cells. The human prostate cancer cell lines PC-3 and DU145 overexpressing EpCAM were purchased from the American Type Culture Collection (ATCC; LGC Promochem). The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium with L-glutamine (L0500, Biowest) supplemented with 10% fetal bovine serum (FBS) (F7524, Sigma-Aldrich; Merck KGaA) and penicillin-streptomycin solution (L0022, Biowest) at 37°C and 5% CO_2 atmosphere, unless stated otherwise. Cells were detached using trypsin-ethylenediaminetetraacetic acid (EDTA) solution (25200056, Thermo Fisher Scientific, Inc.).

Targeting protein. Ec1-LoPE (Fig. 1) was produced and characterized as described previously (38). To confirm the identity, the molecular weight of the protein was determined using electrospray ionization mass spectrometry (Impact II instrument, Bruker Corp.). The spectrometer worked on line with Dionex UltiMate 3000 ultra-high performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Inc.) equipped with a ProSwift RP-4H column (1x50 mm, Thermo Fisher Scientific, Inc.). The chromatographic system used two solvents: Solvent A (3% acetonitrile, 0.1% formic acid in water) and solvent B (95% acetonitrile, 0.1% formic acid in water), and the flow rate was 200 μl/min. The following gradient profile was used: 4% solvent B for 2 min, 4-90% solvent B within 6 min, 90% solvent B for 2 min, 90-4% solvent B within 1 min followed by 4% solvent B for 4 min. The found molecular weight (43,061 kDa) was in excellent agreement with the calculated molecular weight (43,061 kDa) (Fig. 2A). According to HPLC analysis, the purity of Ec1-LoPE was >99% (Fig. 2B).

Radiolabeling. Ec1-LoPE having $(His)_6$-tag at the C-terminus was radiolabeled site-specifically with $[^{99m}Tc][Tc(CO)_3(H_2O)_3]^+$ as described previously (38). Briefly, technetium-99m pertechnetate $[^{99m}Tc]TcO_4^-$ was obtained by eluting a commercial $^{99}Mo/^{99m}Tc$ generator (Mallinckrodt) with sterile 0.9% NaCl. To generate the $[^{99m}Tc][Tc(CO)_3(H_2O)_3]^+$, approximately 500 μl of $[^{99m}Tc]TcO_4^-$ eluate containing 3-4 GBq activity was added to a CRS kit vial (PSI) and incubated at 100°C for 30 min followed by cooling down at room temperature for 10 min. Solution of $[^{99m}Tc][Tc(CO)_3(H_2O)_3]^+$ (100 MBq, 15 μl) was mixed with 30 μl of 0.1 M HCl and added to 30 μg of Ec1-LoPE [25 μl in phosphate-buffered saline (PBS)]. The reaction mixture was incubated for 60 min at 40°C. A pre-purification challenge was performed by adding a 1,000-fold molar excess of histidine

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            10         20         30         40         50
MDLGKKLLEA ARAGQDDEVR ILVANGADVN AYFGTTPLHL AAAHGRLEIV
            60         70         80         90        100
EVLLKNGADV NAQDVWGITP LHLAAYNGHL EIVEVLLKYG ADVNAHDTRG
           110        120        130        140        150
WTPLHLAAIN GHLEIVEVLL KNVADVNAQD RSGKTPFDLA IDNGNEDIAE
           160        170        180        190        200
VLQKAAKLNE FKASGGRHRQ PRGWEQLPTG AEFLGDGGID VSFSTRGTQN
           210        220        230        240        250
WTVERLLQAH AQLEERGYVF VGYHGTFLEA AQSIVFGGVR AASQDLAAIW
           260        270        280        290        300
AGFYIAGDPA LAYGYAQDQE PDAAGRIRNG ALLRVYVPAS SLPGFYRTSL
           310        320        330        340        350
TLAAPEAAGE VERLIGHPLP LALDAITGPE EEGGRLETIL GWPLAERTVV
           360        370        380        390        400
IPSAIPTDPR NVGGDLDPSS IPDKEQAISA LPDYASQPGK PPHHHHHHKD EL
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Figure 1. Primary sequence of the Ec1-LoPE fusion protein. Amino acids constituting the Ec1 domain are marked by bold font.

(110 μg in 11 μl of PBS) to the reaction mixture and incubating at 40˚C for 10 min to remove any loosely-bound $[^{99m}Tc]Tc(CO)_3(H_2O)_3]^+$. The radiolabeled compound was separated from free $[^{99m}Tc]Tc(CO)_3(H_2O)_3]^+$ by passage through a NAP-5 size-exclusion column (GE Healthcare) pre-equilibrated and eluted with PBS. Radiochemical yield and purity of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE were measured using instant thin-layer chromatography (iTLC) silica gel strips eluted in PBS. In this system, the radiolabeled compound stays at the application point; all forms of free radionuclide move with the solvent front. The activity distribution along the iTLC strip was measured with a Storage Phosphor System (CR-35 BIO Plus, Elysia-Raytest) and analyzed with AIDA Image Analysis software (Elysia-Raytest).

In vitro stability test. The evaluation of radiolabel stability of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE was performed by incubating it with a 1,000-fold molar excess of histidine in PBS at room temperature for 4 h. The control samples were incubated in PBS. Samples were analyzed using radio-iTLC in PBS. The values were normalized to the starting radiochemical purity taken as 100%.

In vitro specificity. The binding specificity of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE to EpCAM-overexpressing cells PC-3 and DU145 was performed by using *in vitro* saturation assay (38). Briefly, the cells were seeded in a 6-well plate at a density of $5x10^5$ cells per well one day before the experiment and allowed for attachment overnight. A set of three wells was used for each group. The next day, blocking was performed by incubating one group of cells with 200 nM of non-radiolabeled DARPin Ec1 in 0.5 ml of culture medium at room temperature for 15 min to saturate EpCAM receptors. The same volume of culture medium only was added to another group of cells. After 15 min, 0.5 ml of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE was added to each well to a final concentration of 2 nM and incubated at 37˚C for 1 h. After incubation, medium was discarded, cells were washed once with 1 ml of PBS. The cells were then incubated with 0.5 ml of trypsin solution at 37˚C to allow for detachment and resuspended with 0.5 ml of culture medium. The cell suspension was collected, counted for cell number, followed by a wash with 1 ml PBS, which was also collected. Three standard samples containing 0.5 ml of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE were collected. The activity in standard samples and fractions containing cells was measured using an automatic gamma spectrometer (2480 Wizard, Perkin Elmer), percentage of cell-associated activity per $1x10^6$ cells was calculated.

Cellular processing. Cellular processing and internalization of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE by PC-3 and DU145 cells were evaluated during continuous incubation using acid wash method (39). Briefly, cells were seeded one day before the experiment in 3-cm Petri dishes at a density of $7x10^5$ cells per dish. At the day of the experiment, the radiolabeled compound (2 nM, 1 ml) was added to each dish and the dishes were incubated at 37˚C in a humidified incubator. Three standard samples containing 1 ml of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE were collected and used as total added activity. At 1, 2, 4 and 6 h a set of three dishes was removed, medium was discarded, cells were washed once with 1 ml of ice-cold PBS and discarded. To collect the membrane-bound activity, the cells were incubated with 1 ml of 0.2 M glycine buffer containing 4 M urea (pH 2.0) for 5 min on ice, washed with 1 ml of the same buffer and with 1 ml of PBS. These steps were performed on ice to prevent internalization. To collect the internalized activity, the cells were incubated with 1 ml of 1 M NaOH at 37˚C for 30 min to lyse the cells. The cell lysate was collected, followed by washing with 1 ml of PBS. The activity in the standards, membrane-bound and internalized fractions was measured using a gamma spectrometer and percentage cell-associated activity was calculated.

Affinity. The binding affinity of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE to DU145 cells was measured using LigandTracer Yellow instrument (Ridgeview Diagnostics) as described previously (38). The experiment was performed in duplicate at room temperature to prevent internalization. In brief, $2x10^6$ cells were seeded to a local area of an 89-mm Petri dish one day before the experiment. The binding kinetics was measured by adding increasing concentrations of the radiolabeled compound (3 and 9 nM) every 1.5 h. Thereafter, the medium containing the radiolabeled compound was replaced with cell culture medium to measure the dissociation rate. The binding curve was analyzed using TraceDrawer Software (Ridgeview Instruments, version 1.9) and the equilibrium dissociation constant (K_D) was calculated. The interaction heterogeneity was estimated using Interaction Map analysis (Ridgeview Diagnostics).

Cytotoxicity. The *in vitro* cytotoxicity assay was performed using PC3 and DU-145 cells. Cells were seeded in a 96-well plate at a density of 5,000 cells per well in 100 μl culture medium and allowed to attach overnight. On the experimental day, the medium was removed, serial dilutions of Ec1-LoPE in culture medium were added for each concentration (n=4-5) and the plate was incubated in a humidified incubator. After 72 h, the medium was replaced, and cell viability was evaluated with the Cell Counting Kit-8 (CCK-8; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Briefly, 10 μl of CCK-8 kit was added to each well containing 100 μl culture medium, followed by incubation at 37˚C for 1 to 2 h. The absorbance at 450 nm was measured using a microplate reader. The value from the wells containing medium only was taken as the background; the value from the wells containing cells with
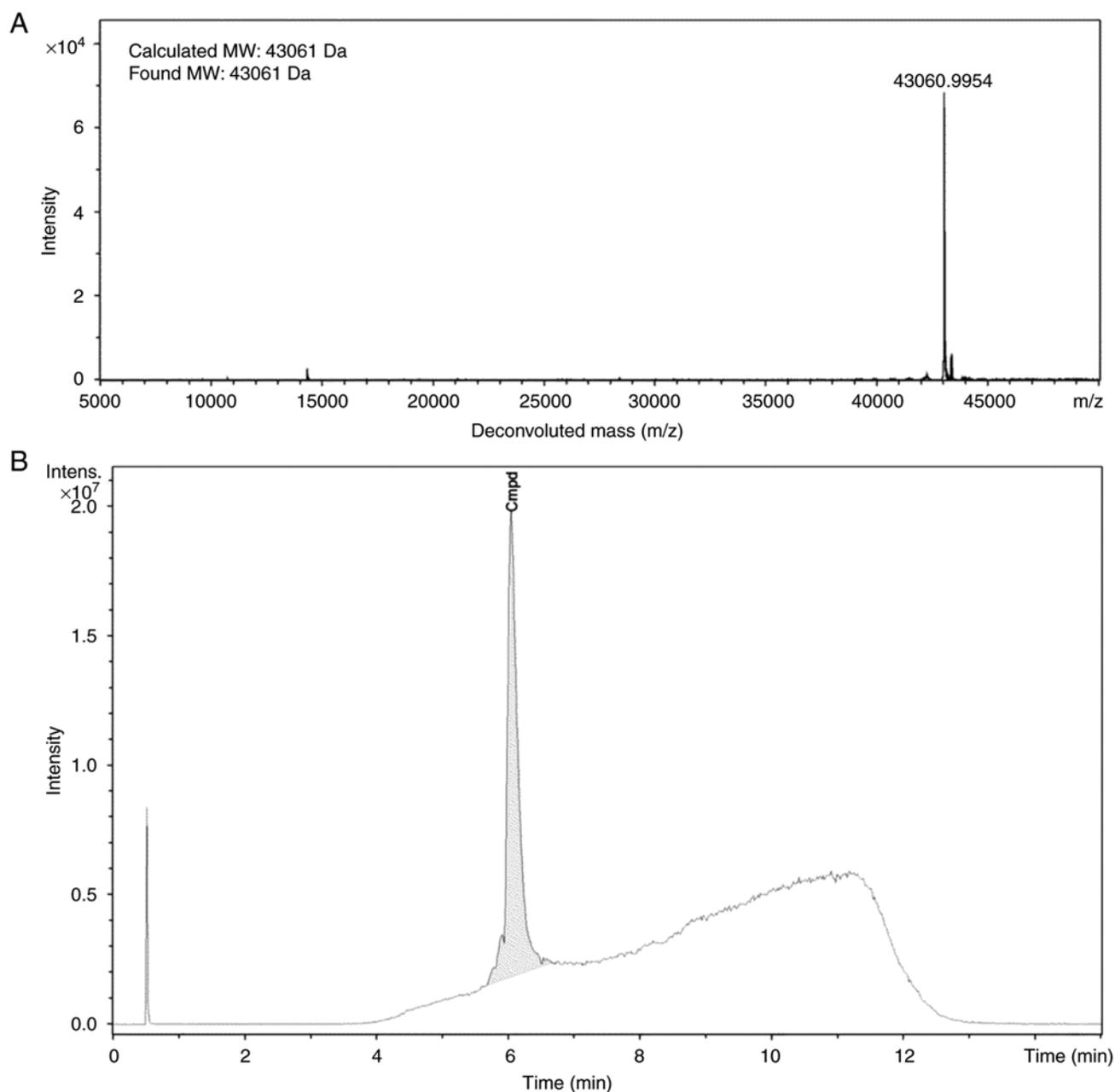
Figure 2. Mass-spectrometry-HPLC analysis of Ec1-LoPE. (A) Deconvolution results from the mass-spectrometry of Ec1-LoPE. (B) HPLC chromatogram of Ec1-LoPE. HPLC, high performance liquid chromatography.

medium (no Ec1-LoPE) was taken as 100% viability control. The data were analyzed by GraphPad Prism (version 9.0.2 for Windows, GraphPad Software, Inc.) using a log(inhibitor) vs. response-variable slope (four parameters) model to obtain a half-maximal inhibitory concentration (IC_{50}) value. To test the specificity of Ec1-LoPE cytotoxic action, one group of cells was incubated with a 100-fold molar excess of DARPin Ec1 for 10 min before the addition of Ec1-LoPE (10 nM) to saturate binding sites on EpCAM and prevent binding of Ec1-LoPE. The cell viability was evaluated as described above.

Statistics. Data were analyzed using GraphPad Prism (version 9.0.2 for Windows, GraphPad Software, Inc.). Unpaired 2-tailed t-test was performed to determine significant differences ($P < 0.05$).

Results

Radiolabeling and stability. Ec1-LoPE was successfully labeled with $[^{99m}Tc]Tc(CO)_3(H_2O)_3]^+$ with a radiochemical yield of 51±13% (n=7) and radiochemical purity over 99% (n=7) after purification using size-exclusion column (Table I). Radiolabeled $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE demonstrated high stability during incubation with a 1,000-fold molar excess of histidine for up to 4 h at room temperature (Table I).

In vitro binding specificity. The specificity of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE binding to EpCAM-expressing PC-3 and DU145 cells was studied by saturating the EpCAM receptors with a large excess of unlabeled DARPin Ec1. A significant ($P < 0.001$) reduction in cell-associated activity was observed

Table I. Radiolabeling of EpCAM-targeting Ec1-LoPE with $[^{99m}Tc]Tc(CO)_3$ and *in vitro* stability under a 1,000-fold molar excess of histidine compared to PBS (control).

			Protein-associated activity (%) (n=2)			
			1 h		4 h	
	Radiochemical yield (%)	Radiochemical purity (%)	x1,000 Histidine	PBS (control)	x1,000 Histidine	PBS (control)
$[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE	51±13 (n=7)	99±0 (n=7)	100±0	100±0	100±0	100±0

Stability analysis was performed in duplicate. Values are normalized to the starting radiochemical purity taken as 100%.

Table II. Equilibrium dissociation constants (K_D) for the interaction between $[^{99m}Tc]Tc$-labeled Ec1-LoPE and EpCAM-expressing DU145 cells.

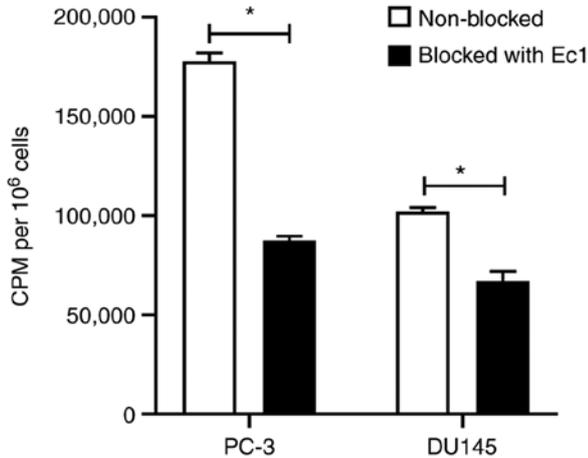|	K_{D1}	K_{D2}
$[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE	2.1±0.3 nM	45±21 nM

Analysis was performed in duplicate.

Figure 3. Binding specificity of $[^{99m}Tc]Tc$-labeled EpCAM-targeting Ec1-LoPE to PC-3 and DU145 prostate cancer cells. For blocking, a 100-fold molar excess of non-labeled Ec1 was added before addition of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE. The final concentration of radiolabeled compound was 2 nM. The data are presented as average ± standard deviation of three samples. *P<0.001. CPM, counts per minute; EpCAM, epithelial cell adhesion molecule.
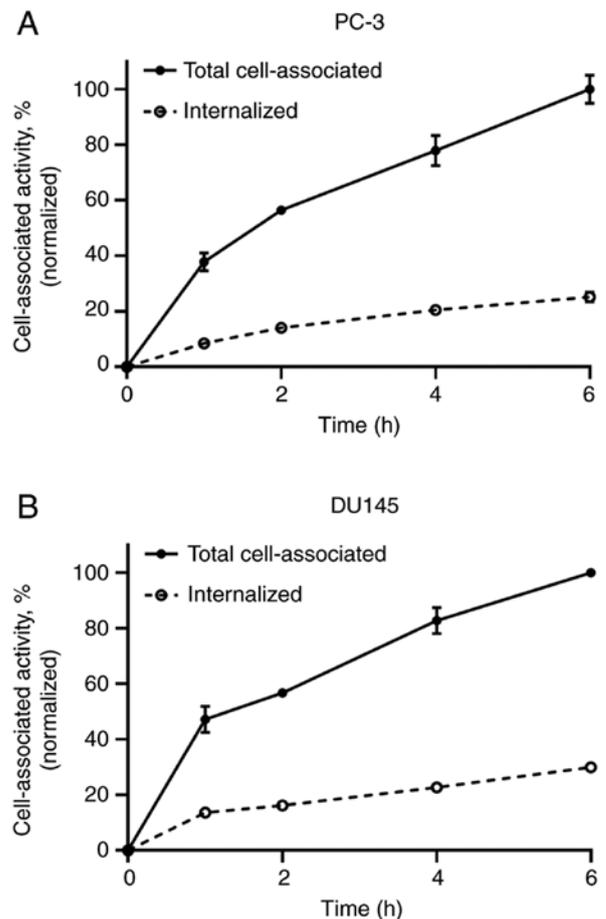

Figure 4. Cellular processing and internalization of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE by EpCAM-expressing (A) PC-3 and (B) DU145 cells during continuous incubation over 6 h. Data are presented as average ± standard deviation of three samples; error bars may not be visible when they are smaller than the symbols used. EpCAM, epithelial cell adhesion molecule.

in the groups where EpCAM was pre-saturated, indicating specific binding of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE (Fig. 3). Cell-associated activity was higher for PC-3 cells compared to DU145 cells.

Cellular processing. The binding of $[^{99m}Tc]Tc$-labeled Ec1-LoPE to both PC-3 and DU145 cells was rapid, and

cell-associated activity was increased with incubation time. The internalized fraction reached 25±2% in the PC-3 cells and 30±0% in the DU145 cells after 6 h of incubation (Fig. 4).

Binding kinetics. The binding of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE to living DU145 cells was characterized by rapid association, while the dissociation included a rapid and a slow phase
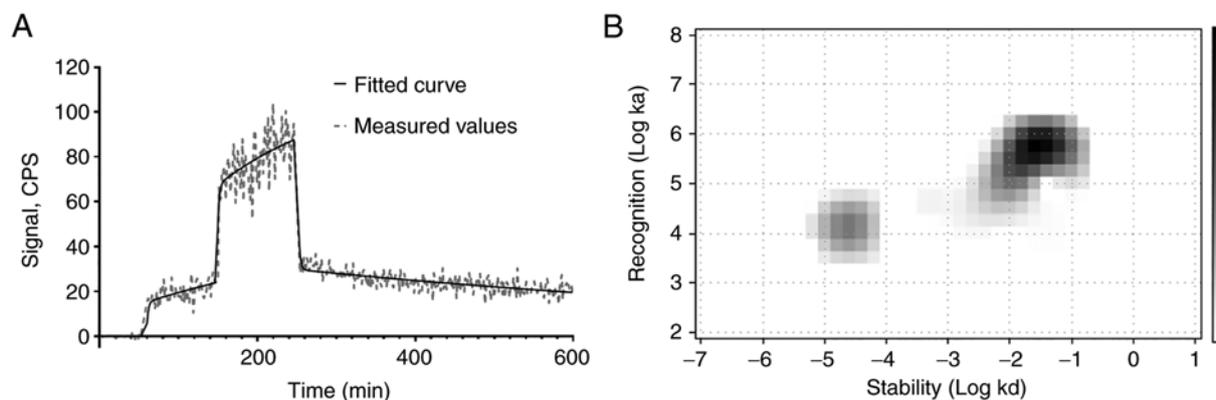
Figure 5. Binding kinetics of [^{99m}Tc]Tc(CO)$_3$-Ec1-LoPE to EpCAM-expressing DU145 cells (A) analyzed by TraceDrawer and (B) a representative Interaction Map. Data are representatives from duplicate. EpCAM, epithelial cell adhesion molecule; CPS, counts per second.
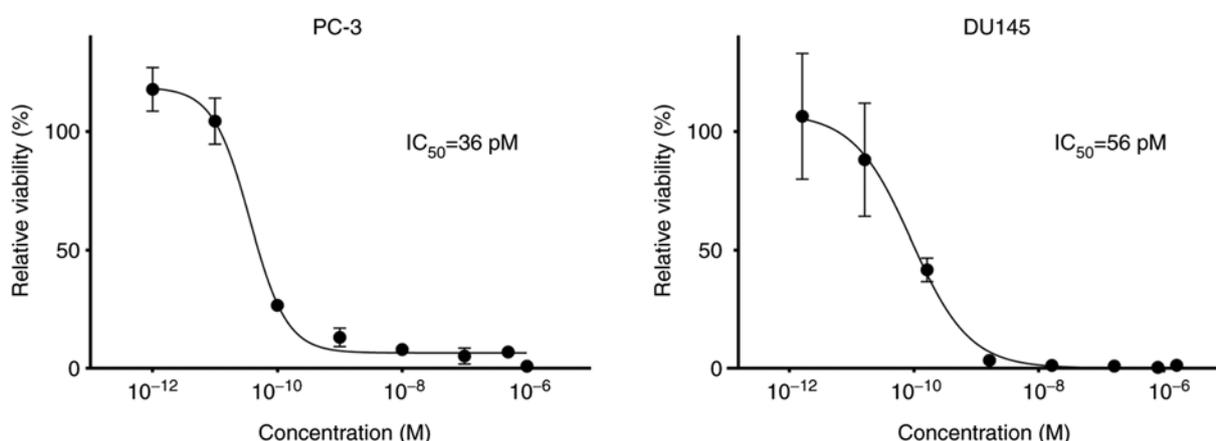

Figure 6. Cytotoxicity of Ec1-LoPE in EpCAM-overexpressing PC-3 and DU145 cell lines. The cells were incubated with the dilution series (0-1,000 nM) of the EpCAM-targeting Ec1-LoPE. The viability of cells grown in medium was used as 100% viability control. The viability curve is plotted with relative viability as the y-axis and concentration of Ec1-LoPE as the x-axis. The data are presented as average ± standard deviation of four to five samples. EpCAM, epithelial cell adhesion molecule; IC$_{50}$, half-maximal inhibitory concentration.
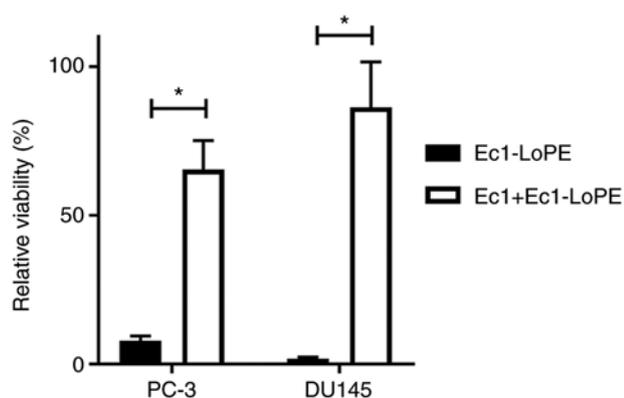

Figure 7. Specificity of Ec1-LoPE cytotoxic action in PC-3 and DU145 cells. In the first group, cells were incubated with Ec1-LoPE only (10 nM). In the second group, cells were incubated with a 100-fold molar excess of designed ankyrin repeat proteins (DARPins) Ec1 for 10 min before the addition of Ec1-LoPE (10 nM). The viability of cells grown in medium was used as 100% viability control. The relative viability data are presented as average ± standard deviation of four to five samples. *P<0.0001.

(Fig. 5). The Interaction Map analysis indicated the presence of two interactions, one with affinity in the low nanomolar range

(K_{D1}=2.1±0.3 nM) and another one approximately 20 times weaker (K_{D2}=45±21 nM) (Table II).

Cytotoxicity. The results of cytotoxicity of Ec1-LoPE on EpCAM-expressing PC-3 and DU145 cells are shown in Fig. 6. Ec1-LoPE demonstrated a dose-dependent cytotoxic effect with subnanomolar IC$_{50}$ values, 36 pM for PC-3 cells and 56 pM for DU145 cells. Pre-incubation of cells with a large excess of DARPin Ec1 before the addition of Ec1-LoPE significantly (P<0.0001) reduced its cytotoxic action (Fig. 7), which suggested that the cytotoxic action of Ec1-LoPE was EpCAM-dependent.

Discussion

Treatment of metastasized prostate cancer (PC) with androgen deprivation therapy (ADT) provides an effective initial response in the majority of patients; however, it is not curative and tumors often develop resistance over the course of treatment. In the next phase of cancer progression into metastatic castration-resistant type, chemotherapy with docetaxel is added to ADT to increase the therapeutic efficacy (40). While providing a cytotoxic effect, conventional chemotherapeutic

drugs do not discriminate between normal and cancer cells resulting in numerous side effects due to cumulative toxicity to normal organs.

Targeted therapeutic agents offer the advantage of reducing cytotoxic side effects on normal cells by directing the payload selectively to cancer cells. The most investigated targeted cytotoxic agents are antibody-drug conjugates, with several of them being approved for clinical use (41). In comparison with monoclonal antibodies (mAbs), small engineered scaffold proteins, such as designed ankyrin repeat proteins (DARPins), might be more effective to overcome biological barriers and deliver a cytotoxic payload to solid tumors, as well as to provide more even distribution in tumors (24). Due to faster clearance from blood and reduced exposure to normal tissues, a larger therapeutic window may also be achieved. Fusion of a DARPin to a bacterial toxin by genetic engineering enables recombinant production of a therapeutic agent as a single protein, removing the need for additional conjugation and purification steps (34). Genetic engineering provides an opportunity for facile optimization of molecular design (e.g., addition of targeting modules or linkers) and biodistribution characteristics.

Examples of scaffold proteins carrying bacterial toxins as a payload include affibody molecules (42), ABD-derived affinity proteins (ADAPTs) (43) and DARPins (44) targeting human epidermal growth factor receptor 2 (HER2). A number of agents have been developed using DARPins targeting EpCAM (Ec1 or Ec4) carrying *Pseudomonas aeruginosa* exotoxin A (ETA'', PE40), which have shown potent antitumor activity in xenograft models of colon cancer, small cell lung carcinoma (45) and breast cancer (46). To address the problem of immunogenic response to the bacterial toxin, B-cell recognition epitopes on the PE toxin were removed (36,47). The new toxin variant termed LoPE was fused to HER2-targeting DARPin 9_29 and showed lower non-specific toxicity and lower immunogenicity *in vivo* than DARPin 9_29-PE40, while preserving a potent cytotoxic action on ovarian cancer xenografts in mice (37). We recently demonstrated that Ec1-LoPE fusion targeting EpCAM caused a significant inhibition of tumor growth, which was additionally potentiated by a combination with HER2-targeting treatment in breast cancer (48) and ovarian cancer (38) models in mice. Repeated administration of Ec1-LoPE was well-tolerated with no observable toxicities or weight loss in mice.

Contrary to the IgG scaffold, the use of small engineered scaffold proteins for the development of targeted anticancer therapeutics is very recent. Therefore, more studies are necessary to improve the understanding of their therapeutic effect in different cancer types. In this study, we used DARPin Ec1 as an EpCAM-targeting module with a molecular weight of approximately 8-fold smaller than an mAb. The addition of LoPE toxin provided Ec1-LoPE fusion protein (43 kDa), which is smaller than a Fab fragment. Overexpression of EpCAM in a large fraction of prostate cancers and in metastatic lesions makes it a possible target for therapy of disseminated PC. This is further supported by a correlation between EpCAM overexpression and increased risk of PC recurrence (10,11).

To obtain quantitative information concerning the functional characteristics of Ec1-LoPE in PC cell lines, we applied site-specific radiolabeling approach using tricarbonyl technetium-99m as a label. Tricarbonyl technetium-99m was attached to the $(His)_6$-tag at the C-terminus of Ec1-LoPE to form a residualizing label, which stays inside the cells after internalization and lysosomal degradation of the protein. This labeling method should have a minimal impact on the binding properties of Ec1-LoPE, since the label and the binding site are spatially separated. Radiolabeling results were in agreement with the previously reported data (38) and the stability of the radiolabel was confirmed (Table I).

The binding of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE to EpCAM-expressing PC-3 and DU145 cells was specific as demonstrated by the saturation experiment. The level of cell-associated activity per cell number was higher for PC-3 cells than for DU145 cells. Massoner *et al* reported that PC-3 and DU145 cells have a similar level of EpCAM expression at both the mRNA and protein levels, while PC-3 cells were found to have a higher degree of EpCAM-positive cells than DU145 by flow cytometry (7), which might be an explanation for the differences in the level of the cell-associated activity.

Previous studies have shown that the binding of Ec1 to EpCAM-expressing cells triggers receptor-mediated endocytosis and enables intracellular delivery of cytotoxic agents (35,48). Cellular processing experiment showed that approximately a quarter of cell-bound activity (25±2%) of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE was internalized by 6 h in PC-3 cells and 30±0% in DU145 cells, which suggests a sufficiently high internalization rate. These results are in line with a previous study in ovarian carcinoma cell lines, where internalization of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE was also rapid, with 31±0% in OVCAR3 cells and 38±3% in SKOV3 cells by 6 h (38).

Analysis of $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE binding kinetics to DU145 cells showed the presence of two interactions, a stronger (K_{D1}=2.1±0.3 nM) and a weaker one (K_{D2}=45±21 nM), both being in the nanomolar range. A similar pattern of two interactions was observed for $[^{99m}Tc]Tc(CO)_3$-Ec1-LoPE binding to SKOV3 and OVCAR3 cells, with the first equilibrium dissociation constant (K_{D1}) being in the subnanomolar range. Comparing these results to DARPin Ec1 alone having K_D of approximately 0.3 nM to DU145 cells (average value for different radiolabel types) (32), it can be concluded that fusion of Ec1 with LoPE resulted in a slight decrease in affinity, which might be expected due to the addition of a bulky toxin moiety to the targeting Ec1 module.

Ec1-LoPE demonstrated a potent cytotoxic effect with IC_{50} values in the low picomolar range in both PC-3 and DU145 cells. High efficiency of cell growth inhibition might be due to a combination of rapid internalization, efficient delivery and high sensitivity to LoPE toxin action. In our recent study, Ec1-LoPE efficiently inhibited tumor growth of SKOV3 xenografts in mice, while having an IC_{50} value of 0.53 μM in SKOV3 cells in the cytotoxicity assay (38). It must be noted that SKOV3 cells were shown to be more resistant to the cytotoxic action of drug (49) and toxin conjugates (50) based on affibody molecules and ADAPTs (43,51) in comparison to other cell lines. Therefore, picomolar IC_{50} values of Ec1-LoPE in both PC-3 and DU145 cells suggest its potential for a therapeutic effect in PC models *in vivo*.

As the efficacy of targeted therapy depends on the presence of a sufficient amount of molecular target in tumors, it

is important to select the patients with a high level of target expression. For the EpCAM-targeting mAb adecatumumab the probability of tumor progression was significantly lower in breast cancer patients who had high EpCAM expression and were treated with a high dose (14,52). High expression of EpCAM was also found to be a precondition for response of PC to adecatumumab (15). In addition to a conventional biopsy-based approach, evaluation of target expression could be performed by positron emission tomography (PET) or single photon emission tomography (SPECT) imaging. Radionuclide molecular imaging is a non-invasive method that provides whole-body information about target expression in real time and can be performed repeatedly to monitor changes in expression or receptor occupancy. The use of a pair of targeting agents, one for diagnostic imaging and another one for targeted therapy, forms a theranostic approach to patient treatment, where imaging is used for stratification of patients for targeted therapy. We recently reported on the preclinical development of Ec1-based radionuclide imaging probes, which efficiently visualize EpCAM expression in PC xenografts (32). Such probes may be used as companion diagnostics in the treatment of PC xenografts with Ec1-LoPE enabling selection of patients that would most likely benefit from such treatment.

In conclusion, DARPin Ec1 is an efficient targeting domain for LoPE toxin. Ec1-LoPE showed EpCAM-specific binding to EpCAM-expressing PC-3 and DU145 PC cells. Rapid internalization mediated potent cytotoxic effect in both studied cell lines. Taken together, these data support the further evaluation of Ec1-LoPE in a therapeutic setting in PC models *in vivo*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TX and YL performed the experiments and analyzed the data. AS and EK performed the production and purification of proteins. SMD participated in the molecular design, supervised the production, purification and characterization of protein, and coordinated the work. VT participated in the study design, labeling chemistry development, data treatment and interpretation, and coordinated the work. AV obtained funding, participated in the study design, labeling chemistry development, data treatment and interpretation, coordinated the work. TX and AV wrote the first version of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work and all data are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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